Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 5351–5355, July 1989
Biophysics

Mechanism of spontaneous vesiculation

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Communicated by John D. Baldeschwieler, February 27, 1989 (received for review August 16, 1988)

ABSTRACT **Both naturally occurring and synthetic phosphatidic acid (PtdOH) molecules show the phenomenon of spontaneous vesiculation on jump in pH value. This method involves a transient increase in pH of smectic PtdOH dispersions to values between 10 and 12. Such a pH increase induces spontaneous vesiculation with the formation of small unilamellar vesicles of diameter <50 nm as shown by ^{31}P NMR. Both high-resolution and broad-line ^{31}P NMR were used to study the mechanism of this process. When the pH of unsonicated PtdOH dispersions is raised to pH 10–12, lipid molecules on the outer monolayer of the bilayer become fully ionized. The second pK value of PtdOH in bilayers is 8.6 ± 0.3, determined by ^{31}P NMR. PtdOH molecules on the inner monolayer remain partially protonated. ^{31}P NMR provides unambiguous evidence that the "pH-jump" treatment produces a pH gradient across the PtdOH bilayer. The orientation of the pH gradient is such that the pH in the external medium is 3–5 pH units higher than the internal pH. Associated with the pH gradient is a transverse packing asymmetry: partially protonated PtdOH molecules in the inner layer of the bilayer are more tightly packed than fully ionized molecules present in the outer layer. The pH gradient generated by the pH jump is proposed as the energy source that drives the spontaneous formation of highly curved vesicles.**

Spontaneous vesiculation is defined as the formation of unilamellar vesicles upon dispersing a dry smectic (lamellar) lipid film in H_2O or raising the pH of a smectic phosphatidic acid (PtdOH) dispersion to values >8 (1–3). The phenomenon of spontaneous formation of vesicles, which implies that no external energy is supplied to the system, is well documented (1–13). Negatively charged lipids with a propensity for smectic phases were shown to disperse in excess H_2O/physiological saline to spontaneously form unilamellar vesicles (1). Similarly, mixtures of zwitterionic phosphatidylcholine (PtdCho) molecules and charged lipids form unilamellar, rather than multilamellar, vesicles when sufficient charged lipid occurs in the bilayer so that the charge density exceeds ≈1–2 $\mu C/cm^2$ (1–5, 8, 11). The unilamellar vesicles formed under these conditions are usually >100 nm. Spontaneous formation of small unilamellar vesicles was described for PtdOH and mixed PtdCho/PtdOH dispersions in 1982 (3). The method used, usually referred to as "pH jump" or pH-adjustment method, involves a quick transient increase in pH to values of 10–12, so that the primary phosphate group of PtdOH becomes fully ionized. Vesicles thus formed are 20–60 nm, similar to the sizes obtained by ultrasonication of phospholipid dispersions. Vesicle size correlates with several experimental parameters including the maximum pH to which the lipid dispersion was adjusted (3, 7, 11), the timing of the pH change (7, 11), the ionic strength of the medium (7, 9, 11), the nature of the phospholipid, and the composition of the phospholipid mixture (1–5, 8, 11–13). Modifications and extensions of the pH-jump method have been described that lead to large unilamellar vesicles (diameter >150 nm) of selected size with rather homogeneous size distribution (10, 11). All these methods require that a charged lipid be at least one component of the lipid bilayer. Recently the phenomenon of spontaneous vesiculation was shown not to be restricted to charged lipids or lipid mixtures. Mixtures of egg PtdCho (ePtdCho) and its lysoderivative underwent spontaneous vesiculation at a certain molar ratio (14). In this case the wedge-shaped molecule of lysophosphatidylcholine was suggested to be responsible for inducing bilayer curvature and spontaneous vesiculation.

The mechanism of spontaneous vesiculation is straightforward for processes leading to unilamellar vesicles with diameters greater than ≈100 nm. As discussed previously (1, 13), this result is a consequence of the infinite swelling behavior of charged mesophases (15–18). In contrast, the mechanism of the spontaneous formation of small unilamellar vesicles with a diameter smaller than ≈60 nm is not yet clear. That small unilamellar vesicles of ePtdCho do not form spontaneously but require an extrinsic source of energy is well known. The energy input is usually provided by ultrasonication. The resulting small ePtdCho vesicles are unstable because they aggregate and fuse over time to large multilamellar structures; the instability was interpreted to indicate that these small vesicles are not in thermodynamic equilibrium (19–21). This paper addresses the mechanism of the spontaneous formation of small unilamellar vesicles (diameter <60 nm). Because these vesicles form spontaneously—that is, without apparent input of energy—they should be thermodynamically stable.

MATERIALS AND METHODS

ePtdCho and egg PtdOH (ePtdOH) were purchased from Lipid Products (Surrey, U.K.) and used without further purification. 1,2-Dilauroyl *sn*-phosphatidic acid, 1,2-dimyristoyl *sn*-phosphatidic acid, 1-myristoyl *rac*-phosphatidic acid (1-myristoyl-*rac*-glycero-3-phosphoric acid), 1-lauroyl *rac*-phosphatidic acid all as the disodium salt, and 1-myristoyl-*sn*-glycero-3-phosphocholine were synthesized by R. Berchtold (Biochemisches Laboratorium, Bern). The phospholipids were pure by TLC standard.

Preparation of Phospholipid Dispersions. Unsonicated phospholipid dispersions in H_2O were prepared as described (1). Sonication of aqueous phospholipid dispersions was done under standard conditions by using a Branson B-30 sonicator with a microtip as described (19). PtdOH samples appeared to be sensitive to degradation, particularly by sonication. Purity checks by TLC and ^{31}P NMR after sonication revealed sample degradation with the formation of mainly lysophosphatidic acid and fatty acids, but occasionally glycerophosphoric acid and other degradation products were detected. Aqueous micellar dispersions of lysophospholipids were prepared by dispersing the solid compound in the appropriate volume of H_2O.

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Abbreviations: PtdOH, phosphatidic acid; PtdCho, phosphatidylcholine; ePtdOH, egg phosphatidic acid; ePtdCho, egg phosphatidylcholine.

pH-Jump Method. Spontaneous vesiculation in unsonicated PtdOH dispersions was achieved by the pH-jump method described in detail previously (3, 6–8). Essentially, the apparent pH of the phospholipid dispersion was raised to pH 11–12 transiently by adding 1 M NaOH either within a few seconds or at least within 2 min and immediately reduced to neutrality by adding 1 M HCl; the latter process was conveniently done within ≈2 min (3, 6, 7). Similar results were obtained by directly dispersing a dry lipid film deposited on the glass wall of a flask in the appropriate NaOH solution to pH 11–12 and neutralizing the alkaline dispersion.

Phospholipid dispersions for ^{31}P high-resolution NMR were made in mixtures of $H_2O/^2H_2O$, 1:1 (vol/vol) for locking purposes; phospholipid dispersions for ^{31}P broad-line NMR studies were made in H_2O. The apparent pH or p^2H is the pH meter reading uncorrected for isotope effects.

Proton-decoupled ^{31}P high-resolution NMR spectra were recorded on a Bruker HXE 90 Fourier-transform spectrometer operating at a ^{31}P frequency of 36.43 MHz. Proton-decoupled ^{31}P powder NMR spectra were run on a Bruker CXP 300 Fourier-transform spectrometer operating at a ^{31}P frequency of 121.47 MHz. Chemical shifts were measured relative to 85% orthophosphoric acid and are expressed as chemical shielding σ—that is, signals upfield with respect to the reference are positive.

RESULTS

The process of spontaneous vesiculation by pH jump is illustrated in Fig. 1. Aqueous unsonicated dispersions of the sodium salt of 1,2-dimyristoyl *sn*-phosphatidic acid ($DMPANa_2$) at pH 7 yield an axially symmetric ^{31}P NMR powder spectrum typical of liquid crystalline bilayers with rapid axial averaging (Fig. 1*A*). The chemical shielding anisotropy $|\Delta\sigma| = |\sigma_{\parallel} - \sigma_{\perp}|$ varied between 48 and 54 ppm ($n = 4$). When the pH of the dispersion was raised to ≈12 with NaOH the ^{31}P powder pattern collapsed to a sharp singlet close to the reference at 0 ppm (Fig. 1*B*). The sharp signal at pH 12 indicates small lipid particles undergoing fast, isotropic tumbling. ^1H NMR revealed these particles to be mainly small unilamellar vesicles (refs. 3 and 7; compare Figs. 2 and 4). Titrating the $DMPANa_2$ dispersion from pH 12 back to pH 8.1 with HCl produced again an axially symmetric powder pattern, $|\Delta\sigma| = 55$ ppm, with a small residual isotropic component (Fig. 1*C*).

Unsonicated dispersions of ePtdOH at pH 2–3 gave a single broad peak of linewidth $\Delta\nu_{1/2} = 500$ Hz rather than the typical powder pattern of unsonicated liquid crystalline bilayers (Fig. 2*A*). Titrating the unsonicated dispersion with NaOH produced a sharp singlet at pH 8.5 (Fig. 2*B*) and a doublet at pH 10.5. The intensity ratio of the downfield and upfield signals was ≈2 (Fig. 2*C*). After addition of enough sodium cholate (≈2%) to solubilize the phospholipid bilayer vesicles to mixed micelles, the doublet collapsed to a single sharp resonance (data not shown). Therefore the two signals probably represent phospholipid molecules in the outer and inner monolayer of the bilayer, respectively. The pH at the outer surface is evidently significantly higher than the pH on the inner surface, causing chemically shifted signals. Upon back titration of the alkaline ePtdOH dispersion to neutrality a sharp singlet appeared similar to that seen in Fig. 2*B*. Reducing the pH of the ePtdOH dispersion further to ≈2 had little effect: a sharp singlet was retained (Fig. 2*D*). The spectral changes described in Fig. 2 *B–D* were reversible.

^{31}P high-resolution NMR spectra of sonicated phospholipid dispersions are presented in Fig. 3. Sonicated ePtdCho dispersions gave a split ^{31}P resonance as has been reported (Fig. 3*A*). The downfield signal was assigned to external phospholipid based on the effect of Co^{2+}; $CoCl_2$ (5 mM) added to the sonicated ePtdCho dispersion broadened the downfield signal beyond detection, consistent with reports (22–25). In contrast to sonicated ePtdCho, sonicated ePtdOH, dilauroyl phosphatidate, and dimyristoyl phosphatidate dispersions at neutral pH gave sharp singlets (Fig. 3 *B–D*), the chemical shift of which was downfield with respect to the ^{31}P signals of ePtdCho. The minor downfield resonance seen with sonicated ePtdOH (Fig. 3*B*) has a chemical shift consistent with lysophosphatidate and is probably a degradation product formed during sonication. The sharp singlets in Fig. 3 *C* and *D* appear superimposed on minor broad signals. The latter are probably from some vesicle aggregation, an interpretation consistent with electron microscopy of freeze-fractured preparations of these two saturated PtdOH dispersions. Electron micrographs reveal largely small unilamellar vesicles and some aggregates.

For comparison, a representative ^{31}P high-resolution NMR spectrum of an unsonicated ePtdOH dispersion is shown (Fig. 4*A*). Spontaneous vesiculation was induced in this dispersion by the pH jump. The relatively good high-

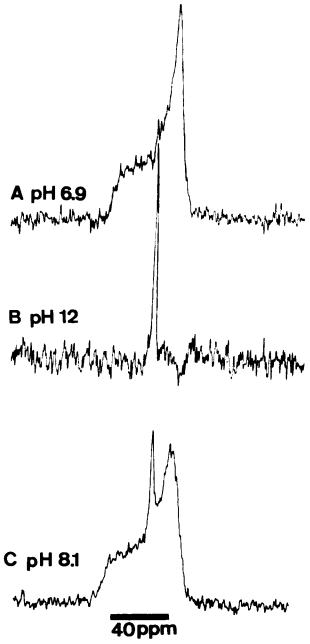

FIG. 1. Proton-decoupled ^{31}P powder NMR spectra of unsonicated, aqueous dispersions of the disodium salt of 1,2-dimyristoyl *sn*-phosphatidic acid at different pH values. ^{31}P NMR spectra were recorded at 121.47 MHz on a Bruker model CXP 300 spectrometer. (*A*) Original dispersion (≈130 mg/ml = 0.2 M) was dispersed in H_2O, pH 6.9. (*B*) This dispersion was made alkaline (≈pH 12) with NaOH. (*C*) The pH of this dispersion was returned to pH 8.1 with HCl. All spectra were recorded at 25°C. The chemical-shielding anisotropy $|\Delta\sigma|$ was ≈50 ppm and 55 ppm in spectra *A* and *C*, respectively. Chemical shielding was measured relative to 85% H_3PO_4.

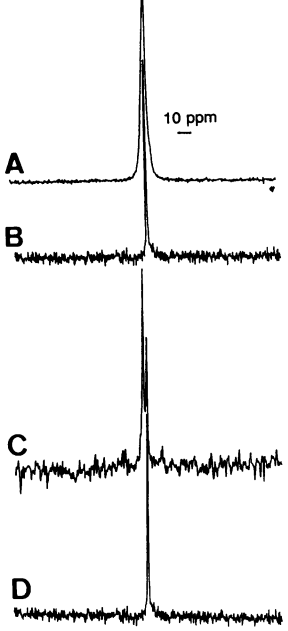

FIG. 2. Proton-decoupled ^{31}P powder NMR spectra of unsonicated, aqueous ePtdOH dispersions at different pH values. ^{31}P NMR spectra were recorded at 121.47 MHz on a Bruker model CXP 300 spectrometer. (*A*) ePtdOH (≈100 mg/ml = 0.15 M) was dispersed in H_2O to pH 2.5. (*B*) In another preparation ePtdOH was dispersed in dilute NaOH, pH 8.5 (43 mg/ml = 0.06 M). (*C*) NaOH was added to the sample of *B* to pH 10.5. (*D*) The pH of sample *C* was adjusted to pH 2 with 1 M HCl. All spectra were recorded at 25°C. Chemical shielding was measured with respect to 85% H_3PO_4.

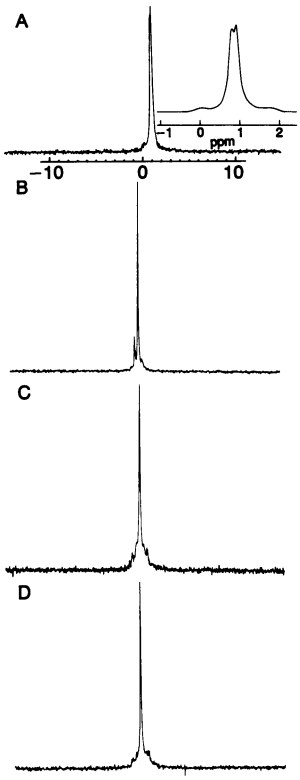

FIG. 3. Proton-decoupled ^{31}P high-resolution NMR spectra of sonicated phospholipid dispersions in $H_2O/^2H_2O$, 1:1 (vol/vol). (*A*) ePtdCho dispersion (20 mg/ml = 0.0267 M), pH 4.0; (*Inset*) expanded spectrum. (*B*) ePtdOH (15 mg/ml = 0.022 M), pH 7.0. (*C*) Disodium salt of 1,2-dilauroyl *sn*-phosphatidic acid (20 mg/ml = 0.034 M), pH 7.3. (*D*) Disodium salt of 1,2-dimyristoyl *sn*-phosphatidic acid (20 mg/ml = 0.031 M), pH 7.2. The phospholipid dispersions were all tip-sonicated. ^{31}P high-resolution NMR spectra were recorded at 25°C and at 36.43 MHz by using a Bruker HEX 90 spectrometer. Chemical shielding is expressed relative to 85% H_3PO_4.

resolution NMR spectrum is consistent with the spectrum (Fig. 2*B*) recorded on the wide-line Bruker CXP-300 instrument, except that the high-field shoulder in Fig. 4*A* was not resolved on the wide-line instrument (Fig. 2*B*). When the pH of the unsonicated ePtdOH dispersion (Fig. 4*A*) was raised again to 11.7, the major signal assigned to external lipid molecules moved further downfield, so that two clearly discernible signals resulted (Fig. 4*B*). Similar changes in chemical shift were seen when the pH of sonicated dispersions of ePtdOH (Fig. 4*C*) and 1,2-dilauroyl phosphatidic acid (Fig. 4*D*) were raised to ≈12. The major resonance assigned to external phospholipid moved downfield, whereas a minor signal arising from internal phospholipid remained at a constant chemical shielding or moved slightly downfield.

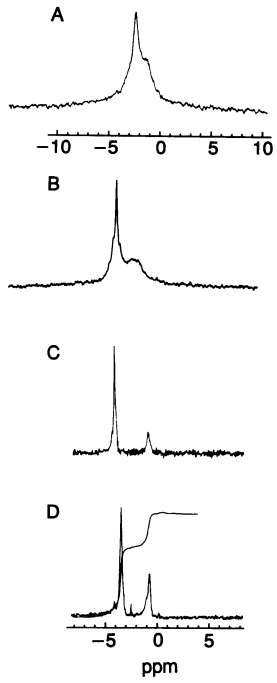

FIG. 4. Proton-decoupled ^{31}P high-resolution NMR spectra of different phospholipid dispersions in $H_2O/^2H_2O$, 1:1 (vol/vol) recorded at 36.43 MHz at 25°C. (*A*) Unsonicated ePtdOH dispersion (33.3 mg/ml = 0.044 M) was subjected to the pH jump: the ePtdOH dispersion was made alkaline (pH 12.6) with 1 M NaOH, and then the pH was returned to 8.1 with HCl. (*B*) The pH of the dispersion in *A* was raised again to pH 11.7 with 1 M NaOH. (*C*) The pH of the sonicated ePtdOH dispersion (20 mg/ml = 0.03 M) was adjusted to 12.1 with 1 M NaOH. (*D*) The pH of the sonicated dispersion of the disodium salt of 1,2-dilauroyl phosphatidic acid (30 mg/ml = 0.052 M) was adjusted to 11.7 with 1 M NaOH. Included in *D* is the integral curve. Chemical shielding is expressed relative to 85% H_3PO_4.

Sonicated dispersions of the disodium salt of 1,2-dimyristoyl *sn*-phosphatidic acid behaved differently. At all pH values tested relatively broad unresolved signals were seen (compare with Fig. 1). The reason for this difference is unknown. The downfield-shift changes induced by raising the pH (compare Fig. 4 *B–D*) were reversible (compare the titration curves in Fig. 5). Adding HCl to the sonicated ePtdOH dispersion (Fig. 4*C*) caused the major downfield signal to move upfield and, by lowering the pH sufficiently (below the starting pH of ≈7), to overtake the stationary minor signal (data not shown).

Fig. 5 shows titration curves of different PtdOH dispersions. With unilamellar vesicles the chemical shielding of the major ^{31}P NMR signal (assigned to external phospholipid) was plotted as a function of pH. For lysophosphatidic acid micelles only one ^{31}P NMR signal was seen over the total pH range. The following conclusions can be drawn from inspecting Fig. 5. There is good agreement between the titration curves of sonicated ePtdOH vesicles and ePtdOH vesicles produced by the pH-jump method. The titration curves of ePtdOH vesicles agree well with those of sonicated dispersions of 1,2-dimyristoyl phosphatidic acid (DMPA) and 1,2-dilauroyl phosphatidic acid. These curves also agree reasonably well with data from DMPA in sonicated mixed ePtdCho/DMPA dispersions of molar ratio 1:1 and 9:1 (data not shown; compare in Table 1). ePtdOH incorporated into micelles of 1-myristoyl lysophosphatidylcholine gave a titration curve slightly shifted to low σ values, at least at pH values >7. The titration curves of lysophosphatidic acids that form micelles are shifted significantly to lower σ values (Fig. 5*B*). The pH titrations were reversible apart from some hysteresis effects (Fig. 5).

The pK values derived from the pH titration are summarized in Table 1. Our values agree well with published data (26–32). The two pK values of the primary phosphate group of PtdOH depend on the state of molecular aggregation. The pK values of PtdOH in bilayers are 3.9 ± 0.1 (pK_1) and 8.6 ± 0.3 (pK_2) (Table 1). For comparison, pK values of *sn*-glycero-3-phosphoric acid were determined in aqueous solutions where this compound is in monomeric form. Both pK values (pK_1 = 2.1 and pK_2 = 6.2) are ≈2 pH units lower than the pK values of PtdOH in bilayers (compare in Table 1 and ref. 26). The two pK values (pK_1 = 3.5 and pK_2 = 7.8) of myristoyl lysophosphatidic acid forming small micelles are also significantly lower compared to PtdOH in bilayers. The value of pK_2 = 8.3 of ePtdOH present in micelles of 1-myristoyl lysophosphatidylcholine is slightly reduced compared to ePtdOH bilayers.

DISCUSSION

One requirement for producing small unilamellar PtdOH vesicles by pH jump is exposure of the dispersion to pH >10. At this pH the primary phosphate group of PtdOH is fully ionized (compare Fig. 5 and Table 1). Small unilamellar PtdOH vesicles produced by sonication give a single ^{31}P signal (Fig. 3 *B–D*). The singlet indicates that (*i*) there is no difference in the molecular packing between outer and inner monolayers and (*ii*) there is no difference in pH between the external medium and the vesicle cavity. In contrast, small unilamellar ePtdCho vesicles produce two ^{31}P signals that are assigned to external and internal ePtdCho molecules (22–25). Packing constraints in the highly curved inner layer of the bilayer have been suggested to be responsible for the second chemically shifted signal.

^{31}P NMR spectra of both sonicated and unsonicated PtdOH dispersions at a pH above ≈10 consist of two ^{31}P signals (Figs. 2*C* and 4): a major downfield signal that can be assigned to external phospholipid present in the outer monolayer and a minor upfield signal assigned to internal phospholipid. The

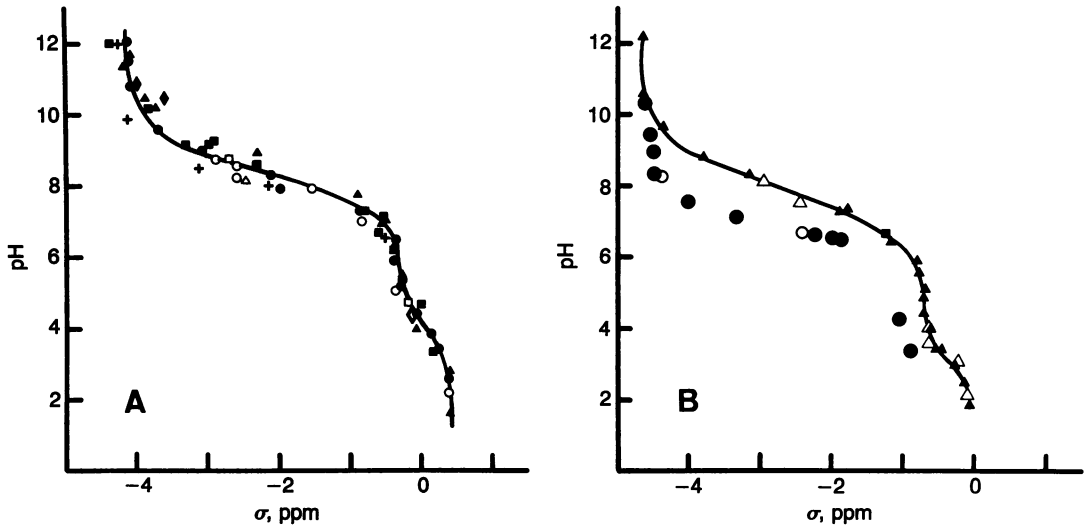

FIG. 5. Titration curves of different aqueous PtdOH dispersions. The chemical shielding (ppm) measured relative to 85% H_3PO_4 is shown as a function of the apparent pH. (*A*) Diacyl phosphatidic acid dispersions: sonicated dispersion of ePtdOH (●, ○), unsonicated dispersion of ePtdOH (33.3 mg/ml = 0.049 M) subjected to pH jump (▲, △), sonicated dispersion of the disodium salt of 1,2-dimyristoyl *sn*-phosphatidic acid (■, □), sonicated dispersion of the disodium salt of 1,2-dilauroyl *sn*-phosphatidic acid at 30 mg/ml = 0.052 M (◆, ◇), ePtdOH dispersed in micelles of 1-myristoyl-*sn*-glycero-3-phosphocholine at 40 mg/ml, pH 6.5; molar ratio, 1:3.3 (+). Closed symbols represent upward titrations—that is, titrations going to higher pH values; open symbols are downward titrations. Unless otherwise stated, the phospholipid concentration was 20 mg/ml. Chemical shielding of the PtdOH samples was independent of concentration over the range of 10–40 mg/ml tested. (*B*) Micellar dispersions of lysophosphatidic acids: the disodium salt of 1-myristoyl *rac*-phosphatidic acid (15 mg/ml = 0.035 M; ▲, △), the disodium salt of 1-lauroyl *rac*-phosphatidic acid (15 mg/ml = 0.038; ●, ○), the disodium salt of 1-palmitoyl *rac*-phosphatidic acid (15 mg/ml = 0.033 M; ■). Closed and open symbols are used to indicate upward and downward titrations, respectively.

assignment is based on titration curves (Fig. 5) and signal intensity measurements. Unsonicated PtdOH dispersions, after being treated by pH jump, yield two ^{31}P signals (Fig. 4 *A* and *B*). From the ^{31}P NMR data, the major downfield resonance can be assigned to external PtdOH molecules; the minor, not completely resolved, signal (Fig. 4*A*) can be assigned to lipid molecules located on the inner surface. The difference in chemical shift apparently reflects differences in the surface pH of the outer and inner monolayer. Therefore, for spontaneous formation of small unilamellar PtdOH vesicles to occur, the generation of a transverse pH gradient is necessary. The orientation of the pH gradient across the PtdOH bilayer is such that the internal pH is more acidic than that of the external medium. The titration curves in Fig. 5 are used to convert chemical shielding to apparent pH. From ^{31}P chemical-shielding measurements it is concluded that the pH-jump treatment produces a pH gradient of 3–5 pH units.

Concerning a mechanism for the spontaneous formation of small unilamellar vesicles the following sequence of events is proposed: upon exposing PtdOH dispersions or packets of dried PtdOH bilayers deposited on the glass wall of a flask to pH 10–12, the external PtdOH molecules on the outer monolayer become fully ionized, whereas internal PtdOH remains partially protonated, thus producing a pH gradient across the PtdOH bilayer. As a result, small unilamellar vesicles bud off spontaneously from large bilayer sheets. This budding process can be rationalized, at least in a qualitative way. PtdOH is a molecule of approximately cylindrical shape (30). Without a pH gradient and any other asymmetry factors, the planar bilayer will be the most stable structure as predicted for cylindrical molecules (33, 34).

However, in the presence of a pH gradient (inside more acidic) a greater electrostatic repulsion will exist between PtdOH molecules in the outer layer than between molecules

Table 1. pK values of different PtdOHs derived from the ^{31}P titration curves in Fig. 5

Sample	pK_1	pK_2	Comments
Sonicated ePtdOH	3.9 ± 0.1	8.6 ± 0.3	Different batches and preparations of ePtdOH; small unilamellar vesicles (n = 4)
Unsonicated ePtdOH	3.8	8.8	Unilamellar vesicles made by pH jump
Sonicated ePtdCho/ePtdOH at 9:1 molar ratio		8.6	Unilamellar vesicles
ePtdOH in mixed micelles		8.3	ePtdOH present in micelles of 1-myristoyl lysophosphatidylcholine
Sonicated 1,2-dimyristoyl phosphatidic acid (disodium salt)		8.5	Unilamellar vesicles
1-Myristoyl *rac*-phosphatidic acid (disodium salt)	3.5	7.8	Micelles
1-Lauroyl *rac*-phosphatidic acid (disodium salt)		7.0	Micelles
sn-Glycero-3-phosphoric acid*	2.1	6.2	Monomeric solution in water

*For comparison ^1H and ^{31}P NMR spectra of *sn*-glycero-3-phosphoric acid (Fluka) dissolved in H_2O were recorded as a function of pH. The ^1H and ^{31}P titration curves yielded pK values that agreed within 0.1 pH unit. The titration curves of *sn*-glycero-3-phosphoric acid and lysophosphatidic acid were used to identify degradation products formed during sonication or on exposing PtdOH dispersions to high pH.

in the inner layer. As shown by monolayer studies a significant expansion in molecular area occurs when the pH is raised from 7 to 12 (35). For instance, at 20°C and a surface pressure of 27 mN/m the area per dihexadecyl phosphatidic acid molecule increases by 6 Å^2 per molecule from 41.3 Å^2 to 47.1 Å^2. This result suggests that a pH gradient across a PtdOH bilayer will create a molecular packing gradient; molecules exposed to lower pH on the internal surface would be more tightly packed. The pH gradient will therefore express itself in a transverse packing asymmetry of the PtdOH bilayer. The sum of the volumes of one external PtdOH molecule and its counterpart on the inner monolayer can be envisaged as wedge shaped—matching the packing requirement of highly curved bilayers. The pH gradient across the PtdOH bilayer may be regarded as the driving force of the spontaneous formation of highly curved and possibly thermodynamically stable vesicles (B. Tenchov, personal communication). We note here that PtdOH does not form micelles at pH 10–12. ^{1}H and ^{31}P NMR spectra as well as freeze-fracture electron microscopy rule out the presence of micelles at pH 10–12, at least to any significant extent. Therefore, the micellization of PtdOH at high pH cannot play a major role in the spontaneous formation of small unilamellar vesicles by the pH-jump method.

According to the mechanism outlined above, the spontaneous formation of small unilamellar vesicles is expected to be a general phenomenon and not restricted to PtdOH and its analogues. In principle, it should be applicable to amphipathic, bilayer-forming molecules with one or more ionizable groups (compare ref. 8). Furthermore, spontaneous formation of small unilamellar vesicles would be predicted to generally occur with two-component or multicomponent lipid mixtures consisting of two types of molecules: (*i*) cylindrical and (*ii*) wedge shaped (B. Tenchov, personal communication). I stress that the spontaneous formation of small unilamellar vesicles is actually a property of lipid mixtures and not of pure phospholipids like ePtdCho. PtdOH is not an exception to this rule. The pH jump generates the two types of molecules required for spontaneous vesiculation: (*i*) the fully ionized, highly hydrated PtdOH that is wedge shaped and preferentially located on the outer monolayer of the bilayer and (*ii*) partially protonated, probably less hydrated, PtdOH that is cylindrical and located on the inner half of the bilayer. For lipid mixtures consisting of differently shaped molecules the pH jump is a possible, but not absolutely necessary, requirement. Spontaneous vesiculation is therefore predicted to occur with mixtures of neutral and isoelectric lipids provided the lipid mixture consists of cylindrically and wedge-shaped molecules in the correct proportion. An example of this type of spontaneous vesiculation has been reported recently (14). Mixtures of ePtdCho of approximately cylindrical shape and its lyso-compound that is wedge shaped have been shown to vesiculate spontaneously, forming small unilamellar vesicles. Furthermore, the molar ratio of the two types of molecules was found to be a critical parameter: spontaneous vesiculation occurs over a very narrow range of molar ratios. Additional lipid mixtures that satisfy the discussed requirements need testing for verification or modification of the hypothesis advanced in this paper.

I acknowledge the help and technical assistance of Jeannette Stäuble. I am indebted to Dr. Boris G. Tenchov for helpful discussions. This work was supported by the Swiss National Science Foundation (Grant 3.223-0.85).

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